

METHOD OF IDENTIFYING MODULATORS OF HIV-1 VPU AND GAG  
INTERACTION WITH U BINDING PROTEIN (Ubp)

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Serial No. 60/083,567, filed April 30, 1998.

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RESEARCH AND DEVELOPMENT

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BACKGROUND OF THE INVENTION

Human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus subfamily of retroviruses. Like all retroviruses, lentiviruses encode the *gag*, *pol*, and *env* genes. However, lentiviruses also contain several accessory genes. The accessory gene *vpu*, which is unique to HIV-1, encodes viral protein U (Vpu) (44). Vpu is a 16-kDa type I integral membrane phosphoprotein that can form oligomeric structures *in vitro* and *in vivo* (32, 43). Indirect immunofluorescence indicates that Vpu localizes predominantly to the Golgi complex (29), but some Vpu is also present in association with the plasma membrane (17). The protein contains a hydrophobic N-terminal domain, which serves as the membrane anchor, and a C-terminal hydrophilic cytoplasmic domain (32).

Vpu plays two roles in HIV-1 replication. First, Vpu promotes the specific degradation of the HIV-1 receptor, CD4, in cell-free systems (8) and *in vivo* (41, 47). Degradation of CD4 enhances the transport and

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subsequent processing of the HIV-1 envelope glycoprotein by releasing it from complexes with CD4 that trap both proteins in the ER (28). Direct interaction of Vpu with the cytoplasmic domain of CD4 is required, but not sufficient, for CD4 degradation (4). Mutational analysis indicates that the hydrophilic cytoplasmic domain of Vpu is required for Vpu-mediated CD4 degradation (40). The second function of Vpu is the enhancement of virus particle release (19, 29, 43, 45, 48). The effect of Vpu on virus particle release appears to be mediated from a post-ER compartment (41). Whereas the cytoplasmic domain of Vpu is important for the degradation of CD4, the transmembrane domain of Vpu is sufficient for partial enhancement of virus release (40). Thus, based on both differential intracellular site of action and genetic criteria the bipartite roles of Vpu are mechanistically distinct. The HIV-1 Gag protein is sufficient for immature virus capsid formation, and those capsids are fully competent for Vpu-mediated enhancement of release, indicating that an eventual target of Vpu during particle release is intrinsic to Gag (30).

The identification of host cell proteins that function in HIV replication has provided crucial insight into the intricacies of the biology of HIV-1. The identification of CD4 as the principal virus receptor on T-cells has provided a basic paradigm for virus entry (10). Chemokine receptors are proteins involved in chemotaxis of immune system cells and have been co-opted

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by HIV-1 to allow entry into host cells in conjunction  
with CD4 (2, 13). Urokinase-type plasminogen activator,  
a proteinase involved in tissue invasion by macrophages,  
binds to and cleaves the HIV-1 envelope glycoprotein  
5 gp120 and enhances the infectivity of HIV-1 in  
macrophages (24). Cyclophilins are proteins that bind to  
the immunosuppressive drug cyclosporin A (CsA) and are  
members of the immunophilin superfamily, which includes  
members that facilitate protein folding (18).  
10 Cyclophilins A, B, and C interact with HIV-1 Gag, and  
cyclophilin A is incorporated into virions (15, 31, 46).  
The incorporation of cyclophilin A into virus particles  
is required for an early step in replication between  
membrane fusion and reverse transcription (5). Furin, a  
15 subtilisin-like endoprotease, mediates the cleavage of  
the HIV-1 envelope glycoprotein precursor gp160 to gp120  
and gp41, a process required for virus infectivity (22).

#### BRIEF SUMMARY OF THE INVENTION

In a preferred embodiment, the present invention is  
20 an assay suitable to determine inhibitors or modulators  
of the Vpu/Ubp interaction. This assay comprises the  
steps of exposing Vpu and Ubp together in the presence of  
a candidate inhibitor under conditions in which Vpu and  
Ubp would normally interact in the absence of the  
25 inhibitor. One then determines whether Vpu and Ubp  
interaction occurs. This assay may be either *in vivo* or  
*in vitro*. A successful inhibitor or compound that alters

Vpu-Ubp interaction is an excellent candidate for an HIV therapeutic. Conversely, in some instances an increase in affinity might negatively affect replication.

The present invention is also an assay for  
5 determining whether a candidate inhibitor inhibits the interaction between Gag and Ubp. This assay comprises the steps of exposing Ubp and Gag in the presence of a candidate inhibitor under conditions in which Ubp and Gag would interact in the absence of the inhibitor. One then  
10 determines whether Gag and Ubp interact. This assay may be either *in vivo* or *in vitro*. As with the assay above, successful inhibitors or compounds that affect interaction would make excellent candidates for anti-HIV therapeutics.

15 In another embodiment the present invention is a preparation of U binding protein (Ubp).

In another embodiment, the preparation is a gene sequence encoding Ubp.

20 In another embodiment, the preparation is an anti-Ubp antibody. This antibody may be either monoclonal or polyclonal.

It is a feature of the present invention that an assay is provided that provides candidates for HIV therapeutics.

25 It is another feature of the present invention that a preparation of U-binding protein and a gene sequence encoding U-binding protein is disclosed.

Other features, objects and advantages of the present invention will become apparent after examination of the specification, claims and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 shows the sequence of the *ubp* coding region and flanking untranslated regions.

Fig. 2A and B illustrates interaction between Vpu and Ubp *in vitro* and *in vivo*. In Fig. 2A a GST-Ubp fusion protein and GST alone were expressed and purified as described in Materials and Methods. In Fig. 2B lysates of mock-transfected HeLa cells (lane 1) or cells transfected with either a vpu- (pBG135, lane 2) or a vpu+ (pGB108, lane 3) proviral DNA construct were subjected to anti-Ubp immunoaffinity column chromatography analysis as described in Materials and Methods.

Fig. 3A is a Northern blot analysis of *ubp* RNA from human cells. Fig. 3B is an *in vitro* translation of Ubp. The plasmid KT173 was used to express Ubp in a coupled transcription/translation system (TNT, Promega) in the presence of  $^{35}$ S-methionine and  $^{35}$ S-cysteine, and protein was analyzed by SDS-PAGE and detected by phosphorimage analysis. Fig. 3C demonstrates endogenous expression and overexpression of Ubp in HeLa cells.

Fig. 4 diagrams a comparison of Ubp with other members of the TPR family. In Fig. 4A Ubp is shown aligned with four proteins resulting from the BLAST

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sequence similarity search. Fig. 4B shows alignment of TPR motifs in Ubp and related proteins.

Fig. 5 illustrates the interaction between GST-Ubp and Gag expressed in bacteria and in HeLa cells. In Fig. 5A the left panel shows a lysate of *E. coli* expressing His-Gag protein analyzed by Western blot using anti-Gag antiserum. In the right panel, an in vitro binding assay was performed as described in Materials and Methods using His-Gag (the total input is shown in the left panel) and either GST-Ubp (lane 2) or GST alone (lane 1). In Fig. 5B the left panel shows anti-Gag Western blot analysis of lysates of HeLa cells transfected with either a vpu- (lane 1) or a vpu<sup>+</sup> (lane 2) HIV-1 proviral construct. The results are shown in the right panel of Fig. 5B (lane 3, vpu- construct; lane 4, vpu<sup>+</sup> construct).

Fig. 6 illustrates the effect of Ubp overexpression on HIV-1 particle release. Fig 6A illustrates a Western blot analysis that was performed on cell lysates with antigen affinity-purified anti-luciferase (top panel) and antigen affinity-purified anti-Ubp (bottom panel) antibodies. Fig. 6B illustrates a particle release assay using a p24 antigen-capture ELISA as described in Materials and Methods.

Fig. 7 is a model of the roles of Vpu and Ubp in particle release.

DETAILED DESCRIPTION OF THE INVENTION

1. In General

Viral protein U (Vpu) is a protein encoded by HIV-1 that promotes the degradation of the virus receptor, CD4, and enhances the release of virus particles from cells.

We isolated a cDNA that encodes a novel cellular protein that interacts with Vpu *in vitro*, *in vivo*, and in yeast. This Vpu-binding protein (Ubp) has a molecular weight of 41-kDa and is expressed ubiquitously in human tissues at the RNA level.

Ubp is a novel member of the tetratricopeptide repeat (TPR) protein family containing four copies of the 34-amino-acid TPR motif. Other proteins that contain TPR motifs include members of the immunophilin superfamily, organelle targeting proteins, and a protein phosphatase.

Ubp also interacts directly with HIV-1 Gag protein, the principal structural component of the viral capsid. However, when Vpu and Gag are coexpressed, stable interaction between Ubp and Gag is diminished.

Furthermore, overexpression of Ubp in virus-producing cells resulted in a significant reduction in HIV-1 virion release. Taken together, these data indicate that Ubp plays a role in Vpu-mediated enhancement of particle release.

In one embodiment, the present invention is a preparation of Ubp, a gene sequence encoding Ubp or an antibody specific for Ubp. In another embodiment, the present invention is a method of detecting interaction

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between either Vpu and Ubp or Gag and Ubp. These methods would be useful to identify inhibitors or modulators of the Vpu/Ubp or Gag/Ubp interaction and, thus, identify potential anti-HIV therapeutics.

5    2. Ubp and Gene Sequence

In one embodiment, the present invention is a preparation of Ubp. The Examples below describe a method of preparing Ubp involving the isolation of a gene sequence coding Ubp. However, one of skill in the art 10 would know of alternative methods of isolating the protein. For example, one might isolate it from bacterial, baculovirus or mammalian cell preparations.

The present invention is also an anti-Ubp antibody and methods of using anti-Ubp antibodies, such as in 15 detection kits or assays to detect Ubp. This antibody may be either monoclonal or polyclonal. The Examples below describe one Ubp antibody. However, other methods known to those of skill in the art would be equally suitable for antibody preparation.

20    In another embodiment, the present invention is a gene sequence encoding Ubp. The Examples below, Fig. 1 and SEQ ID NO:1 disclose the sequence of the Ubp coding region as it naturally occurs. The coding region starts at nucleotide 39 and ends at nucleotide 1187.

25    One may manipulate this gene sequence and still obtain Ubp. For example, one may make nucleotide substitutions without changing the encoded amino acid. Additionally, one may make small mutations, deletions or

additions to the protein and still obtain a characteristic Ubp molecule. The Examples below describe the characteristic properties of Ubp.

One may use the Ubp sequence in the following manner:

A. One may generate small peptide derivatives or fragments that affect Vpu-Ubp interaction or Gag-Ubp interaction. These peptides are potential inhibitors.

B. Ubp could be a member of a superfamily of related proteins. Thus, it is likely that other members of such a superfamily could be detected using nucleic acid probes derived from the human Ubp sequence. Other members of the superfamily would likely have functions related to Ubp and could, therefore, be significant.

C. The human Ubp sequence could be used to isolate Ubp homologs from other species. These homologs may have functions that are similar or related to that of human Ubp.

D. Ubp affects particle release of HIV-1. It is possible that Ubp is a component of an export pathway that is common to that of other retroviruses, or even other families of viruses. If so, by interfering with Ubp in cells that are producing other viruses, it might also be possible to interfere with the export of those viruses.

E. Ubp is involved in efficient particle release from cells. It might be possible to use Ubp to enhance

the release of particles to increase the titer of HIV vectors or other retrovirus vectors.

The assays described in sections 3 and 4, below, are representative of assays that can be used in the present invention to determine whether a candidate inhibitor has an affect on the interaction between either Vpu and Ubp or Gag and Ubp. Inhibitors or compounds that alter the affinity between Ubp and Upu or Ubp and Gag would then be excellent candidates for evaluation as anti-HIV therapeutics.

10 3. Assays to Detect Interaction between Vpu and Ubp

A. In vitro Interaction

Vpu is synthesized in reticulocyte extracts, and Ubp is synthesized in *E. coli* as a fusion protein, such as Ubp-Gst. The two proteins are then incubated together, and the complexes are isolated preferably using glutathione beads (which interact with the Gst portion of the fusion protein). The protein is stripped from the column, and Vpu is then detected by gel electrophoresis.

20 The principle advantage of this system is that it allows rapid and straightforward examination of mutants and reagents that affect protein-protein interaction.

B. Interaction in Yeast

Vpu is synthesized as a fusion protein, preferably with the DNA binding domain of GAL4, while Ubp is expressed as a fusion protein, preferably with the activation domain of GAL4. When coexpressed in yeast, these two proteins associate with each other by virtue of

Vpu-Ubp interaction. This results in the juxtaposition of the GAL4 activation domain with promoters driving indicator genes or selectable markers. Thus, interaction is detected by monitoring the indicator genes or by

5 selection.

This is a viable system for examining the effect of mutants in Vpu or Ubp on Vpu-Ubp interaction. However, it is not as rapid or convenient as the *in vitro* system.

C. Interaction *in vivo*

10 Human cells expressing HIV proteins including Vpu are subjected to lysis. To detect interaction between Vpu and endogenously expressed Ubp, the lysate is then passed through a column containing antibody raised against Ubp. Thus, Ubp is retained on the column and Vpu 15 is also retained by virtue of interaction with Ubp. The protein can then be recovered from the column and Vpu is detected by Western blot analysis with anti-Vpu antisera.

The main advantage of this system is that the system 20 itself is the normal biologically relevant system. Thus, affects on Vpu-Ubp interaction in the system are highly significant.

4. Assays to Detect Interaction between Gag and Ubp

A. In vitro Interaction

25 Gag is synthesized in reticulocyte extracts, and Ubp is synthesized in *E. coli* as a fusion protein, such as a Ubp-Gst fusion. The two proteins are then incubated together, and the complexes are isolated using

glutathione beads (which interact with the Gst portion of the fusion protein), the protein is stripped from the column. Gag is then detected by Western blot analysis using anti-Gag antibody.

- 5       The principle advantage of this system is that it allows rapid and straightforward examination of mutants and, potentially, reagents that affect protein-protein interaction.

B. Interaction In Vivo

- 10      Human cells expressing HIV proteins including Gag are subjected to lysis. To detect interaction between Gag and endogenously expressed Ubp, the lysate is then passed through a column containing antibody raised against Ubp. Thus, Ubp is retained on the column and Gag is also retained by virtue of interaction with Ubp. The protein can then be recovered from the column and Gag is detected by Western blot analysis with anti-Gag antiserum.
- 15

20      5. An assay to examine the effect of Ubp on particle release

- 25      High-level expression of Ubp leads to interference of particle release. We think this is due to competitive interference of Vpu by excess Ubp. This assay would be useful for examining the effect of Vpu, Ubp, and Gag derivatives on particle release itself.

EXAMPLES

1. In General

We used a yeast two-hybrid system to screen a B-lymphocyte cDNA expression library for cellular proteins capable of interacting with Vpu. The principal cDNA that resulted from this screen is a novel cDNA which encodes a 41-kDa protein that is widely expressed on the mRNA level in human tissues. The protein contains four copies of a 34-amino acid repeat motif called the tetratricopeptide repeat (TPR). The TPR family of proteins is composed of proteins of very diverse function including organelle targeting proteins (11, 25), proteins involved in mitosis (26, 42), immunophilins (6, 36), and a nuclear phosphatase (7). Our results indicate that this novel Vpu-binding protein (Ubp) functionally interacts with both Vpu and Gag. Ubp appears to be an intermediary between Vpu and Gag and likely plays a role in virus assembly or release.

2. Materials and Methods

DNA constructions. To construct pKT106, the *vpu* gene was amplified from pGB107 (16), a derivative of the HIV-1 infectious molecular clone pNL4-3 (1), using the primers *vpu1* (5'-AGTAGTACATCATATGCAACCTA-3') (SEQ ID NO:3) and *vpu2* (5'-TCCACACAGGGATCCCCATAAT-3') (SEQ ID NO:4). The 308-bp amplification product was gel purified and digested with *Nde*I and *Bam*HI and cloned into the plasmid pAS1-CYH2 (a derivative of pAS1 (12) containing a cycloheximide sensitivity gene) that had also been cut

with *Nde*I and *Bam*HI. pVpui9-1 is a two-hybrid system library plasmid containing the *ubp* cDNA with a complete 5' end. Sequence analysis of five *ubp* cDNAs indicated that the cDNA from pVpui9-1 is missing 47 bp of the 3' untranslated region including the polyadenylation signal and the poly-A tail. The sequence in Fig. 1 is derived from the cDNA insert of pVpui9-1 plus the 47 bp of the 3' end from the other cDNAs. pKT173 was constructed by cloning a 1412-bp *Xho*I-*Pst*I fragment of pVpui9-1 containing the entire *ubp* coding region into pCITE-2a (Novagen) cut with *Xho*I and *Pst*I. pKT199, which expresses Vpu in a coupled *in vitro* transcription/translation system, was created by inserting a 310-bp *Nde*I-*Bam*HI fragment from pKT106 into pCITE-2a cut with *Nde*I and *Egl*II. pGEX-Ubp was created by inserting a 2204-bp *Xho*I fragment from pVpui9-1 into the *Sal*I site of pGEX-4T-1 (Pharmacia). pHIV-Ubp, which expresses Ubp from the HIV-1 LTR, was created by cloning a 1705-bp *Xho*I-*Nru*I fragment from pVpui9-1 containing the entire *ubp* coding region into pBG139 (19) that had been cut by *Sal*I and *Stu*I. pJL90 was constructed by amplifying the gag gene from pNL4-3 with the primers 1 (5'-CGGGATCCGGTGCAGAGCGTCGGTATTAAAG-3') (SEQ ID NO:5) and 2 (5'-GCTCTAGACCTGTATCTAATAGAGCTTC-3') (SEQ ID NO:6). The PCR product was gel purified and digested with *Bam*HI and *Xba*I and inserted into pQE30 (Qiagen) that had been cut with the same two enzymes. pHIVTF(stop), which contains a mutation that creates a premature stop codon

immediately following the second amino acid of the trans-frame shift peptide, was constructed by PCR amplification of pMSMΔEnv2 (33) with the sense primer 23233 (5'-GGCCAGATGAGAGAACCAAGG) (SEQ ID NO:7) and the antisense mismatch primer 8264 AfIII (5'-CAAAGAGTCAGTTAAGGGAAAGCTAAAG) (SEQ ID NO:8). A second fragment was derived from the PCR amplification of pMSMΔEnv2 with the antisense primer 23234 (5'-CCTATAGCTTATGTCCGCAG) (SEQ ID NO:9) and the sense mismatch primer 8265 AfIII (5'-CTTAGCTTCCCTTAAGTCACTCTTG) (SEQ ID NO:10). These fragments were digested with AfIII, ligated, and reamplified with the primers 23233 and 23234. The amplified fragment was digested with SpeI and BcII, and this 922-bp fragment was ligated into pMSMΔEnv2 that had also been digested with SpeI and BcII. pHJ121 was constructed by introducing the SalI-NheI fragment of pBG135 (19) containing the mutated vpu gene into pHIVTF(stop) that had also been cut with SalI and NheI.

Two-hybrid system library screen. The yeast strain Y190 (MAT<sub>a</sub> leu2-3,112, ura3-52, trp1-901, his3 -D200, ade2-101, gal4D gal80D URA3::GAL-lacZ, LYS2::GAL-HIS3, cyh<sup>r</sup>) was first transformed to Trp prototrophy with pKT106, which expresses Vpu fused to the GAL4 DNA-binding domain. Cells containing this plasmid were grown in Trp-minus synthetic complete (SC) medium and transformed with a human B-lymphocyte cDNA library cloned into a plasmid called pACT (12), which expresses the various

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cDNA-encoded proteins as hybrids with the GAL4 transcriptional activation domain. Transformations were performed as previously described (39). Doubly-transformed yeast were grown on SC medium lacking His, Leu, and Trp (37) and containing 25 mM 3-amino-1,2,4-triazole (Sigma), and yeast colonies that grew were subjected to an X-gal colony filter assay (12). The cDNA-containing plasmids from His<sup>+</sup> colonies that stained blue in the X-gal assay were isolated and tested for their ability to activate transcription alone, and those that did were discarded as false positives. Positive candidate clones were analyzed by restriction digestion and sequencing.

In vitro protein binding assays. For the GST-Ubp/Vpu binding assay, GST and a GST-Ubp fusion protein were expressed in *E. coli* using the plasmids pGEX-2T and pGEX-Ubp, respectively. The proteins were purified using Glutathione-Sepharose (Pharmacia) according to the manufacturer's directions. Protein concentration was quantified using the Bradford assay (Bio-Rad). Vpu was expressed from the plasmid pKT199 using the TNT rabbit reticulocyte lysate *in vitro* transcription/translation system (Promega) according to the manufacturer's directions. Reactions were performed in the presence of 23 mCi Tran-35<sup>s</sup>-label (ICN) to radioactively label the protein. Multiple 25- $\mu$ l reactions were performed and pooled after the incubation period. Twenty  $\mu$ l of pooled Vpu was mixed with 30 pmol

of either GST alone or GST-Ubp, and the binding reactions were brought to a total volume of 200  $\mu$ l with binding buffer (50-200 mM NaCl, 20 mM Tris-HCl pH 7.9, 1 mM EDTA, 5% glycerol, 0.02% NP-40, 2  $\mu$ g/ml leupeptin, 100  $\mu$ g/ml PMSF, 0.05% BSA). Binding reactions were incubated on a rocking platform at 4°C for 2 hours and then at room temperature for 1 hour. A 25- $\mu$ l bed volume of Glutathione-Sepharose beads was added to each reaction, and the reactions were incubated for an additional 2 hours at room temperature on a rocking platform. Beads were washed 3X in 1 ml binding buffer and resuspended in 30  $\mu$ l standard SDS-PAGE protein sample buffer. Samples were heated in a boiling water bath for 4 minutes, spun at 14,000 rpm in a microcentrifuge for 5 minutes, and loaded onto a SDS/15% polyacrylamide gel.

For the GST-Ubp/Gag interaction assay using Gag expressed in bacteria, his-tagged HIV-1 Gag precursor and a GST-Ubp fusion protein were expressed in *E. coli* using the plasmids pJL90 and pGEX-Ubp, respectively. Protein was induced from bacterial expression plasmids with isopropyl-β-D-thiogalactopyranoside according to standard methods (38). Bacteria were pelleted 2 hours after induction, washed with TEK buffer (20 mM Tris-HCl, 100 mM KCl, and 1 mM EDTA, pH 7.4) once and then resuspended in lysis buffer (10 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40, pH 7.4). The bacterial pellets were frozen and thawed and sonicated four times for 15 seconds each.

Insoluble material was pelleted by centrifugation for 10 minutes in a microfuge at 14 K rpm. Total protein concentrations in the supernatants were determined by the Bradford dye-binding procedure (Bio-Rad). Supernatants 5 were adjusted to 20% glycerol and stored at -80°C.

His-tagged Gag protein (approximately 0.1  $\mu$ M) was incubated with GST-Ubp fusion protein or GST protein (approximately 0.5  $\mu$ M) in 300  $\mu$ l Triton buffer (50 mM Tris-HCl, 300 mM NaCl, and 0.5% Triton X-100, pH 7.4) at 10 4°C on a rocking platform for one hour. After incubation, a 50- $\mu$ l bed volume of Glutathione-Sepharose beads (Pharmacia) was added to each reaction, and the 15 incubation was continued for another half hour.

Glutathione-Sepharose beads were pelleted by a 10-second centrifugation in a microfuge and then washed three times with 500  $\mu$ l Triton buffer. Washed Glutathione-Sepharose beads were resuspended with 20  $\mu$ l 4X protein sample buffer (8% SDS, 50 mM Tris, 40% glycerol, 24.8 mg/ml DTT, 0.4% bromophenol blue, 5% 2-mercaptoethanol, pH 6.8), 20 adjusted to a final volume of 80  $\mu$ l, and then heated in a boiling water bath for 5 minutes. After the beads were pelleted in a microfuge, the supernatants were subjected to SDS-PAGE and Western blot analysis using anti-Gag polyclonal antiserum.

25 For the GST-Ubp/Gag binding assays using Gag expressed in HeLa cells, cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) containing 7% calf serum. One day before transfection, 0.8 million

cells were seeded onto 100-mm dishes. HeLa cells were transfected by calcium phosphate coprecipitation followed by glycerol shock with either a vpu+ protease- proviral construct (pHIVTF(stop)) or a vpu<sup>r</sup> protease- proviral construct (pHJ121). Forty-eight to sixty hours post-transfection, cells were washed once with phosphate-buffered saline and lysed with 150 µl Triton lysis buffer (0.5% Triton X-100, 50 mM Tris, 300 mM NaCl, pH 7.5). After incubation on ice for 30 minutes, lysates were spun at 7000 rpm for 6 minutes to pellet cell debris, and supernatants were transferred to new tubes and stored at -20°C. Gag concentrations were determined using a p24 antigen-capture ELISA kit (Coulter). Conditions for the binding assay were the same as those described above for the binding assay with bacterially-expressed Gag protein.

Immunoaffinity column chromatography. An immunoaffinity column was constructed using Affi-Gel 10 (Bio-Rad) and purified IgG from rabbit serum raised against His.Ubp. IgG was coupled to the matrix in coupling buffer (0.1 M HEPES, pH 8.0) at 4°C for 4 hours, placed in a glass column, and washed extensively with column wash buffer (10 mM sodium phosphate, pH 6.8). HeLa cells were washed 2X with PBS then lysed with cell lysis buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl, 10 mM iodoacetamide, 0.5% Triton X-100, 0.2 mM PMSF, 0.5 mM leupeptin). Cell lysates were placed on the column by gravity flow, and the column was washed with ten bed

volumes of wash buffer. Bound protein was eluted with 100 mM glycine pH 2.5 and 1-ml fractions were collected. Fractions containing protein, determined by O.D. 280 nm, were pooled and concentrated to a 100- $\mu$ l volume using

5 Centricon concentrators (Amicon Inc.). These fractions were then analyzed by Western blotting for Ubp and Vpu.

Northern blotting. Twenty-five ng of either a 2-kb human b-actin cDNA or the 2204-bp *Xho*I fragment of pVpu19-1 were labeled with  $a^{32}P$ -dCTP (Amersham) using a

10 random primed labeling kit (Boehringer Mannheim) according to the manufacturer's directions.

Unincorporated nucleotides were removed from the reactions using Sephadex G-50 columns (Boehringer Mannheim). All of the labeled probe from each labeling 15 reaction was used in separate hybridization reactions to probe a multiple human tissue Northern blot (MTN Blot II, Clontech) according to the manufacturer's directions.

#### Antibody production and purification.

Histidine-tagged Ubp, His-tagged HIV-1 Vpu cytoplasmic

20 domain, and His-tagged HIV-1 Gag proteins were expressed in *E. coli* and purified under denaturing conditions with Ni-NTA agarose (Qiagen) according to the manufacturer's directions. The protein samples were dialyzed overnight against 1X PBS. Dialyzed His-tagged proteins were used 25 to raise polyclonal antiserum in rabbits. Crude anti-Gag serum was used for Gag Western blots. IgG was purified from anti-Ubp or anti-Vpu rabbit serum using DEAE Affi-Gel blue (Bio-Rad) or protein-A agarose (Bio-Rad)

according to the manufacturer's directions. For Ubp Western blots, anti-Ubp antibodies were further purified with His-Ubp immobilized on Ni-NTA agarose.

Western blotting. For detection of Ubp or Vpu in 5 mammalian cells, cells were lysed in NP-40 lysis buffer (100 mM NaCl, 20 mM Tris pH 7.9, 1 mM EDTA, 0.5% NP-40) and subjected to low-speed centrifugation in a microcentrifuge to remove cellular debris. Cleared cell lysates were mixed with standard SDS-PAGE protein sample 10 buffer and were subjected to electrophoresis on a SDS/15% polyacrylamide gel. Immunoaffinity column samples were electrophoresed on a SDS/10-20% polyacrylamide gradient gel. Proteins were transferred to Immobilon-P membrane 15 (Millipore) using a Mini Trans-Blot electroblotting apparatus (Bio-Rad) in transfer buffer (27.2 mM Tris, 192 mM glycine, 20% methanol) for 2 hours at 150 mA constant current at 4°C. Membranes were blocked overnight in blocking buffer (5% dry milk, 20 mM Tris, 0.01% NaN<sub>3</sub>). Membranes were then incubated in blocking buffer 20 containing a 1:2000 dilution of an IgG-purified or an antigen affinity-purified rabbit polyclonal Ubp antibody or an IgG-purified rabbit polyclonal Vpu antibody for four hours at room temperature. Membranes were washed 3X in wash buffer (20 mM Tris, 100 mM NaCl, 0.3% Tween-20, 25 0.005% NaN<sub>3</sub>) and were subsequently incubated in blocking buffer containing a 1:10,000 dilution of an alkaline phosphatase conjugated goat anti-rabbit IgG (g-chain specific) antibody (Sigma) for two hours at room

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temperature. Membranes were washed as before and then incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma) for 15 minutes at room temperature.

5        Western blots for luciferase were performed as described above except that a 1:5,000 dilution of an antigen affinity-purified anti-luciferase rabbit polyclonal antibody (Promega) was used for the primary incubation.

10      For the GST-Ubp/Gag binding assay, proteins from a polyacrylamide gel were transferred to a nitrocellulose membrane using a Trans-Blot Cell electroblotting apparatus (Bio-Rad). The membrane was blocked in blocking buffer (5% dry milk, 3% BSA, in PBS) for 40 minutes and then incubated in PBS containing 5% dry milk and 1% BSA and a 1:200 dilution of a rabbit polyclonal anti-Gag antibody for one hour. The membrane was washed in 1x wash buffer (0.05% Tween-20 in PBS) for 20 minutes and incubated in PBS containing 5% dry milk and 1% BSA and a 1:2500 dilution of a 35S-labeled goat anti-rabbit IgG antibody. Phosphorimage analysis was used for quantitative measurement of Western blots.

25      DNA sequencing and sequence analysis. Partial sequencing of cDNAs was performed using a Sequenase dideoxynucleotide sequencing kit (United States Biochemical). The longest *ubp* cDNA from pVpu19-1 was sequenced using an automatic sequencing apparatus (Applied Biosystems). Sequence similarity searches of

the databases were conducted using the NCBI BLAST e-mail server (3). Exact positions of sequence motifs were determined using the MacVector sequence analysis program (International Biotechnologies) and manual analysis.

5 Sequence identity and similarity was calculated manually or with the Bestfit program from the Wisconsin Genetics Computer Group.

p24 antigen-capture ELISA particle release assay.

Triplet cultures of  $5 \times 10^5$  HeLa cells were transfected

10 one day after plating by calcium phosphate coprecipitation with one  $\mu\text{g}$  of either pGB108 (an env-derivative of the HIV-1 molecular clone NL4-3, (16)) or pBG135 (a vpu- derivative of GB108, (19)) and 14  $\mu\text{g}$  of either pHIV-Ubp or pTAR-luc, a plasmid that expresses 15 firefly luciferase from the HIV-1 LTR. Thirty-six hours after transfection, aliquots of medium were collected and subjected to low-speed centrifugation to remove cell debris, and supernatants were transferred to new tubes.

Cells were washed in PBS and scraped into 1 ml PBS and 20 transferred to a centrifuge tube. Cells were pelleted in a microfuge at 6000 rpm for five minutes, and the cell pellets were lysed in 50  $\mu\text{l}$  NETN buffer (0.5% NP-40, 1 mM EDTA, 20 mM Tris pH 8.0, 100 mM NaCl). Lysates were subjected to low-speed centrifugation, and supernatants 25 were transferred to new tubes. The amounts of p24 antigen in medium samples and cell lysates were measured using a p24 antigen-capture ELISA (Coulter Corporation).

### 3. Results

Interaction between Vpu and a novel cellular protein. Two lines of evidence are consistent with the interaction between Vpu and one or more cellular factors.

5 Vpu can enhance significantly the release of retrovirus particles as divergent as HIV-2, visna virus, and Moloney murine leukemia virus (21), albeit to a lesser extent than HIV-1 particles. In addition, Vpu-mediated enhancement of virus release is cell-type dependent. Vpu 10 is required for efficient release of virus particles from some cells, such as HeLa cells and CD4+ T-lymphocyte cell lines, but not from other cells, such as COS-1 or CV-1 monkey kidney cells (20) or Balb/c murine lymphoblastoma cells (23).

15 To identify cellular proteins that might play a role in the activities of Vpu, a yeast two-hybrid system (14) was initially used to screen a human, CD4-negative, lymphocyte cDNA expression library for proteins that interact with Vpu. The two-hybrid system is based on the 20 juxtaposition of the DNA binding and transcriptional activation domains of the yeast transcription factor GAL4. Vpu was expressed as a fusion protein with the GAL4 DNA-binding domain (DBD), and proteins encoded by the cDNA library were expressed as fusions with the GAL4 25 activation domain. If a particular cDNA-encoded protein interacts with Vpu, then the two domains of GAL4 are brought into close association, and GAL4 function is restored. This causes the activation of two

GAL4-responsive reporter genes integrated into the yeast chromosomes: the *his3* gene, which confers on the yeast the ability to grow on media lacking histidine, and the *E. coli lacZ* gene, which causes the colonies to stain blue in an X-gal colony filter assay.

1.5 million yeast transformants were screened in the two-hybrid system for interaction with Vpu, and 13 His+, β-gal+ clones were obtained. Ruling out false positives on the basis of activation of transcription in the

absence of GAL4-DBD-Vpu and other criteria narrowed these down to five candidate cDNA-expressing plasmids. Partial sequence analysis and restriction enzyme analysis indicated that the five cDNAs contain various lengths of the same sequence. The plasmid containing the longest cDNA, designated pVpui9-1, was also tested in the two-hybrid system with plasmids encoding fusion proteins between the GAL4 DNA-binding domain and two proteins unrelated to Vpu: murine TNF receptor associated factor 2 (TRAF-2) and human ADP ribosylation factor 1 (ARF-1). The protein encoded by the cDNA did not bind to TRAF-2 or ARF-1, indicating that the interaction between Vpu and the protein expressed from pVpui9-1 is specific in yeast (data not shown). Fig. 1 shows the sequence of the *ubp* coding region and flanking untranslated regions. The complete nucleotide sequence of the longest *ubp* cDNA obtained from the two-hybrid system Vpu screen is shown with the deduced amino acid sequence of the open reading frame. Numbers to the left of each line designate

nucleotide positions. The four TPR motifs are underlined, and the polyadenylation signal is double-underlined.

A search of sequence databases found matches only to  
5 partial cDNA sequences of unknown function (accession  
numbers Z13137 and D58427), indicating that this is a  
novel cDNA sequence. We have termed this sequence and  
the protein it encodes Ubp, for Vpu-binding protein. The  
ubp coding region is 1146 base pairs long, corresponding  
10 to a 382-residue protein with a predicted molecular  
weight of 41.25 kDa. The ubp mRNA contains an  
exceptionally long 3' untranslated region of 987 base  
pairs. As discussed in more detail below, Ubp is a  
member of the tetratricopeptide repeat (TPR) protein  
15 family and contains four TPR motifs.

To verify the Vpu/Ubp interaction demonstrated with  
the two-hybrid system, an *in vitro* binding assay was  
performed. Fig. 2 illustrates interaction between Vpu  
and Ubp *in vitro* and *in vivo*. In Fig. 2A a GST-Ubp  
20 fusion protein and GST alone were expressed and purified  
as described in Materials and Methods. Protein  
concentrations were measured using the Bradford assay  
(Bio-Rad). Equimolar amounts of GST-Ubp (lane 3) or GST  
alone (lane 2) were incubated in solution with  $^{35}$ S-labeled  
25 *in vitro*-translated Vpu. GST or GST-Ubp was recovered on  
Glutathione-Sepharose beads, and Vpu was detected by  
SDS-PAGE and phosphorimage analysis. Lane 1 shows 1% of  
the input Vpu used in the binding reactions. In Fig. 2B

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lysates of mock-transfected HeLa cells (lane 1) or cells  
transfected with either a vpu- (pBG135, lane 2) or a vpu+  
(pGB108, lane 3) proviral DNA construct were subjected to  
anti-Ubp immunoaffinity column chromatography analysis as  
5 described in Materials and Methods. Eluates from  
immunoaffinity columns are shown in lanes 4-6 (lane 4,  
mock; lane 5, pGB108; lane 6, pBG135). Cell lysates and  
column fractions were subjected to Western blot analysis  
with either anti-Ubp (top panel) or anti-Vpu (bottom  
10 panel) antiserum. His-tagged *E. coli*-expressed Vpu is  
shown in lane 7.

Ubp was expressed as a fusion protein with a portion  
of the enzyme glutathione-S-transferase (GST). The  
GST-Ubp fusion protein was then tested for its ability to  
15 bind to *in vitro*-translated  $^{35}$ S-labeled Vpu (Fig. 2A). At  
75 mM NaCl, Vpu bound to GST-Ubp (lane 3) at a level  
significantly over the background of GST alone (lane 2)  
as measured by phosphorimage analysis. The sensitivity  
of this Vpu-Ubp interaction to salt concentration varied  
20 slightly from experiment to experiment, but the highest  
fold binding over background was always in the range of  
50-100 mM NaCl (data not shown).

The binding of Vpu and Ubp was verified *in vivo*  
using an immunoaffinity column constructed with the  
25 anti-Ubp antibody. pGB108 (16) is an env- derivative of  
the HIV-1 infectious molecular clone NL4-3. pBG135 (19)  
is a vpu- derivative of GB108. HeLa cell lysates,  
representative of mock (lane 1, M), pBG135 (lane 2,

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Vpu-), or pGB108 (lane 3, Vpu+) transfected, were placed on the column. Proteins eluted from the column are shown in lanes 4-6 in both panels (lane 4, mock; lane 5, pGB108; lane 6, pBG135). As expected, eluates from all three conditions showed an enrichment for Ubp when analyzed by Western blotting (Fig. 2B, top panel, lanes 4-6). When the immunoaffinity column fractions were probed for Vpu, a distinct band appeared in only one lane corresponding to those cells transfected with pGB108 (Fig. 2B, bottom panel, lane 5). Neither Ubp nor Vpu bound to an immunoaffinity column made with preimmune rabbit serum, showing that the purification of Vpu by the anti-Ubp column was due to specific interaction with Ubp (data not shown). These results indicate that Ubp and Vpu stably interact *in vivo*.

To identify human cell types that express *ubp* RNA, we performed Northern blot analysis of RNA from eight human tissues including spleen, thymus, prostate, testis, ovary, colon, small intestine, and peripheral blood leukocytes, using a *ubp* cDNA probe. Fig. 3A is a Northern blot analysis of *ubp* RNA from human cells. The top panel shows mRNA from human spleen (lane 1), thymus (lane 2), prostate (lane 3), testis (lane 4), ovary (lane 5), small intestine (lane 6), colon (lane 7), and peripheral blood leukocytes (lane 8), probed with a *ubp* cDNA probe. The bottom panel shows the same blot when stripped and reprobed with a *b*-actin control probe. Fig. 3B is an *in vitro* translation of Ubp. The plasmid KT173

was used to express Ubp in a coupled transcription/translation system (TNT, Promega) in the presence of  $^{35}$ S-methionine and  $^{35}$ S-cysteine, and protein was analyzed by SDS-PAGE and detected by phosphorimage analysis. Fig. 3C demonstrates endogenous expression and overexpression of Ubp in HeLa cells. Because high level expression of Ubp from pHIV-Ubp is Tat-dependent, the plasmid pHIV-Ubp was cotransfected with a plasmid that expresses HIV-1 Tat (pGB108 (16)). Lysates of

5 mock-transfected HeLa cells (lane 1), HeLa cells transfected with pGB108 alone (lane 2), and HeLa cells transfected with pHIV-Ubp and pGB108 (lane 3) were subjected to Western blot analysis using an IgG-purified rabbit polyclonal anti-Ubp antibody.

10

15 The results of this experiment indicated that *ubp* RNA is expressed in each of these tissues (Fig. 3A). Moreover, the length of the detected RNA (2600 nt) was consistent with the length of the *ubp* cDNA (2221 bp) taking into consideration the addition of an

20 average-sized poly-A tail to the *ubp* mRNA.

To determine whether the *ubp* open reading frame could be translated to produce a protein of the expected size, we constructed pKT173, a plasmid which expresses the *ubp* coding region from a phage T7 promoter. pKT173  
25 was used to program *in vitro* coupled transcription/translation reactions. As shown in Fig. 3B, a protein of about 42 kDa was produced. To ensure that the *ubp* cDNA contained the entire coding region, we

constructed pHIV-Ubp, a plasmid that expresses the *ubp* cDNA from the HIV-1 LTR. This plasmid was used to overexpress Ubp in HeLa cells. Cells were lysed at forty-eight hours post-transfection, and the lysates were subjected to Western blot analysis with a rabbit polyclonal Ubp antiserum. As shown in Fig. 3C, two Ubp species of slightly different mobility on the gel were produced in cells transfected with pGB108 and pHIV-Ubp (lane 3), and those two species comigrated with two endogenous UBP species in mock-transfected (lane 1) and GB108-transfected (lane 2) cells. Thus, the *ubp* cDNA appears to contain a full-length open reading frame. Western blot analysis on lysates from COS-1 and CEM (a CD4+ T-cell line) cells indicated that Ubp was also expressed in both of these cell types (data not shown). The genesis of the two species of Ubp in HeLa cells is not clear. The larger of the two species may be the result of post-translational modification.

Alternatively, the smaller of the two Ubp species may be a stable degradation or proteolytically processed product of Ubp. In addition, two minor bands of 25 to 28 kDa are detectable in lysates of HIV-Ubp-transfected cells. While the origin of these species is not known, the bands most likely represent degradation products of Ubp. They are more easily detected in HIV-Ubp-transfected cells because of the high level of expression of Ubp.

Ubp is a novel member of the TPR family of proteins.  
The deduced amino acid sequence of Ubp was used to search

sequence databases for polypeptides that are similar to Ubp. The results of this search identified a group of very diverse proteins including a protein of unknown function from *C. elegans*, a protein of unknown function from *S. cerevisiae*, two immunophilins (CyP-40 (36) and FKBP59 (6)), two organelle targeting proteins (Pxrlp (11) and MAS70 (25)), a nuclear serine/threonine phosphatase (PP5 (7)), and proteins involved in mitosis (nuc2+ (26) and CDC23 (42)). A comparison of Ubp to four of these proteins is shown in Fig. 4A.

Fig. 4 diagrams a comparison of Ubp with other members of the TPR family. (A) Ubp is shown aligned with four proteins resulting from the BLAST sequence similarity search (3). TPR motifs are shown as numbered white boxes. Regions outside the TPR motifs that show sequence similarity to Ubp are shown in black. Regions that do not contain sequence similarity to Ubp are cross-hatched or shaded. (B) Alignment of TPR motifs in Ubp and related proteins. The amino acid sequences of the TPR motifs of Ubp, *C. elegans* R05F9.10 (accession number U41533), *S. cerevisiae* UNF346 (accession number U43491), human PP5, and human CyP-40, are shown aligned with each other and with the TPR consensus sequence. In the consensus sequence, an asterisk indicates any large hydrophobic residue, and a dash indicates any residue. Sequence identity to Ubp is indicated by black reverse print. Sequence similarity to Ubp is indicated by gray shading.

Most of the proteins resulting from the search contain multiple copies of a 34 amino acid long repeat motif called the tetratricopeptide repeat (TPR) (26, 42). Ubp contains four TPR motifs, three of them in tandem (Fig. 1 and Fig. 4A). Similarity between Ubp and most of the proteins identified from the search is almost exclusively limited to the TPR motifs. However, the proteins of unknown function from *C. elegans* and *S. cerevisiae* contain extended regions of similarity to Ubp including sequences outside the TPR motifs, indicating that these proteins are likely to be homologs of human Ubp. The *C. elegans* protein is 45% identical and 63% similar to Ubp with six gaps in the alignment. The *S. cerevisiae* protein is 37% identical and 56% similar to Ubp with eight gaps in the alignment (data not shown). An alignment of the TPR motifs of Ubp with the TPR motifs of other proteins and the TPR consensus sequence (26) is shown in Fig. 4B. The TPR motifs of Ubp match the consensus sequence as well or better than the TPR motifs of previously published TPR proteins.

The TPR family of proteins contains members with very diverse activities, and the motif has been well characterized physically. The TPR motif probably adopts a secondary structure consisting of a 25-30-amino acid amphipathic  $\alpha$ -helix followed by a short proline-induced turn. Formation of this structure has been demonstrated by circular dichroism and limited proteolysis for the protein nuc2+ of *S. pombe* (26). These  $\alpha$ -helices are

believed to interact intra- or intermolecularly with each other through their hydrophobic faces. The TPRs are likely to be involved in mediating interactions between proteins. Indeed, the TPR motifs of the immunophilin  
5 FKBPs9 (34), the peroxisome targeting protein Pxrlp (11), and a mouse homolog of the nuclear phosphatase PP5 (9) have been shown to be involved in protein-protein interaction.

Interaction between Ubp and HIV-1 Gag. The HIV-1  
10 Gag protein is the principal component of the virus particle. Since Gag expression is sufficient for particle formation and responsiveness to Vpu, Gag contains a direct or indirect target of the particle release enhancement activity of Vpu (30). We used  
15 multiple approaches to determine whether Vpu and Gag interact directly and have obtained no evidence for such an interaction. However, an alternative possibility is that the ability of Vpu to enhance particle release is manifested through cellular protein intermediates such as  
20 Ubp. To determine whether Ubp interacts with Gag, we performed an *in vitro* binding assay using these two proteins. His-tagged HIV-1 Gag precursor, a GST-Ubp fusion protein, and GST alone were expressed in parallel cultures of *E. coli*, and bacterial lysates were used for  
25 the *in vitro* binding assay. His-tagged Gag was tested for interaction with either GST-Ubp or GST alone.

Fig. 5 illustrates the interaction between GST-Ubp and Gag expressed in bacteria and in HeLa cells. (A) The

left panel shows a lysate of *E. coli* expressing His-Gag protein analyzed by Western blot using anti-Gag antiserum. In the right panel, an *in vitro* binding assay was performed as described in Materials and Methods using His-Gag (the total input is shown in the left panel) and either GST-Ubp (lane 2) or GST alone (lane 1). Gag protein from the binding assay was also detected using Western blot analysis with anti-Gag antiserum. Total protein concentrations of bacterial lysates used in the binding assay were measured by the Bradford assay. (B)

The left panel shows anti-Gag Western blot analysis of lysates of HeLa cells transfected with either a vpu- (lane 1) or a vpu+ (lane 2) HIV-1 proviral construct. These lysates were used for *in vitro* binding assays as described in Materials and Methods, and the results are shown in the right panel (lane 3, vpu- construct; lane 4, vpu+ construct). Gag from HeLa cell lysates did not bind to GST alone (lanes 5 and 6). The presence or absence of Vpu expression in HeLa cells is indicated above the gels.

As shown in Fig. 5A, HIV-1 Gag protein specifically bound to GST-Ubp (lane 2) at a level significantly over the background of GST alone (lane 1) as measured by phosphorimage analysis. The association between Gag and Ubp could be observed at salt concentrations ranging from 1 mM to 500 mM NaCl or KCl (data not shown). A concentration of 300 mM NaCl was found to be optimal for specific binding. In addition, preliminary results with anti-Ubp immunoaffinity column chromatography indicate

that Ubp interacts with Gag in HeLa cells (data not shown).

Gag protein expressed in transfected HeLa cells was also tested for its ability to interact with GST-Ubp.

- 5 HeLa cells were transfected with either pMS156, a vpu-  
protease- HIV-1 proviral construct, or pHJ121, a vpu-  
protease- proviral construct (30). We examined the  
efficiency of particle release from cells transfected  
with these constructs and observed the expected effect:  
10 particle release was more efficient in the presence of  
Vpu (data not shown). Transfected cell lysates were then  
used as a source of Gag protein for the *in vitro* binding  
assay. When Gag was expressed in the absence of Vpu, Gag  
stably interacted with GST-Ubp (Fig. 5B, lane 3).  
15 However, when Gag was expressed in the presence of Vpu,  
Gag was unable to interact with excess GST-Ubp,  
indicating that the coexpression of Vpu abrogated stable  
UBP-Gag interaction (Fig. 5B, lane 4). To see whether  
Vpu from transfected cells was interacting with GST-Ubp  
20 *in vitro*, thereby preventing interaction between Gag from  
transfected cells and GST-Ubp, Gag and Vpu were expressed  
in separate cell cultures, and lysates were mixed and  
then added to GST-Ubp. The result of this experiment was  
that Gag was able to bind to GST-Ubp (data not shown).  
25 This result demonstrates that Vpu is not simply binding  
GST-Ubp *in vitro* and competitively inhibiting interaction  
between GST-Ubp and Gag. Therefore, the negative effect  
of Vpu on the ability of Gag to bind Ubp is occurring

within HeLa cells. Taken together, these results indicate that Gag may be modified in some way in cells expressing Vpu and that this modification renders Gag unable to interact subsequently with Ubp. It is also  
5 worth noting that preliminary results with anti-Ubp immunoaffinity column chromatography indicate that Ubp interacted with Gag in HeLa cells only in the absence of Vpu.

Effect of Ubp Overexpression on HIV-1 particle

10 release. To determine whether Ubp affects Vpu-mediated enhancement of virus release, Ubp was overexpressed in virus-producing cells in the presence and absence of Vpu expression. As a control for non-specific effects of protein overexpression on particle release, luciferase  
15 was expressed in cultures that were not overexpressing Ubp. Fig. 6 illustrates the effect of Ubp overexpression on HIV-1 particle release. HeLa cells were mock-transfected (M) or transfected with either pGB108 (lanes 1 and 2, Vpu+) or pBG135 (lanes 3 and 4, Vpu-) and  
20 either pHIV-Ubp (lanes 2 and 4, UBP+) or pTAR-luc (lanes 1 and 3, luc+). (A) Thirty-six hours post-transfection, Western blot analysis was performed on cell lysates with antigen affinity-purified .  
anti-luciferase (top panel) and antigen affinity-purified  
25 anti-Ubp (bottom panel) antibodies. Lane numbers above blots correspond to numbers under bar graph in part B.  
(B) Particle release was assayed using a p24 antigen-capture ELISA as described in Materials and

Methods. The data are represented as the ratio of extracellular to intracellular p24 and are normalized to the GB108+TAR-luc cotransfection (bar 1). The data represent one of two independent experiments performed in triplicate. Similar results were obtained from both experiments.

Overexpression of luciferase and Ubp were confirmed by Western blot analysis using the appropriate antisera (Fig. 6A). As expected, the lack of wild-type Vpu expression resulted in a five-fold reduction in particle release (Fig. 6B, compare bars 1 and 3). In the presence of Vpu, Ubp overexpression caused a four-fold decrease in virus release (Fig. 6B, compare bars 1 and 2). In the absence of Vpu, particle release was further reduced two- to three-fold by Ubp overexpression (Fig. 6B, compare bars 3 and 4). The intracellular p24 levels in cells overexpressing Ubp were not reduced compared to cells expressing luciferase (data not shown), indicating that the inhibition of particle release by Ubp overexpression was not due to a cytotoxic effect of high levels of Ubp. These results demonstrate that overexpression of Ubp in virus-producing cells has a negative effect on HIV-1 particle release.

#### 4. Discussion

The fact that high level expression of Ubp reduces the efficiency of particle release suggests a simple negative role for Ubp. Moreover, the observation that Vpu forms stable complexes with Ubp and abrogates stable

Ubp-Gag interaction is consistent with the idea that association of Ubp with Gag is detrimental to virus release, and that a role of Vpu is to dissociate Ubp-Gag complexes. However, scrutiny of the data from Fig. 5 provides an intriguing alternative possibility. Stable association between Gag and Ubp is detected only in the absence of Vpu; when Vpu is present, only stable Vpu-Ubp complexes are observed. The fact that no detectable Gag is found associated with GST-Ubp when Gag is expressed in the presence of Vpu suggests that the interaction between Ubp and Gag, along with subsequent dissociation by Vpu, leads to an irreversible change in Gag resulting in the inability of Gag to interact with Ubp. In this scenario, Ubp would be a factor required for correct particle formation and release. Overexpression of Ubp would negatively affect particle release simply by competitively inhibiting Vpu: association between excess free Ubp and Vpu would interfere with the ability of Vpu to dissociate Ubp-Gag complexes.

A possible model for Vpu-mediated enhancement of HIV-1 virion release is presented in Fig. 7. Fig. 7 is a model of the roles of Vpu and Ubp in particle release. Ubp interacts with Gag forming a complex. In the absence of Vpu, this complex is stable and may be inhibitory to viral particle release. When Vpu is present, Ubp is disassociated from Gag allowing for modification(s) of Gag to form Gag\*. This modification changes the protein in such a way that it can no longer interact with Ubp.

Either the inability of Gag\* to interact with Ubp or the modification of Gag itself results in enhancement of virus release. Ubp interacts with Gag resulting in the transient formation of Gag-Ubp complexes (in the absence 5 of Vpu, these complexes are stable). Vpu then interacts with Ubp resulting in dissociation of Gag-Ubp complexes. Since the resulting Gag is no longer competent for binding to GST-Ubp, it appears that interaction of Gag with Ubp and subsequent Vpu-mediated dissociation results 10 in an irreversible modification of Gag (in the figure, this modification is indicated by Gag\* for heuristic purposes). This uncharacterized modification of Gag may influence Vpu-mediated particle release in one of two ways. First, the conversion of Gag to Gag\* may be 15 directly responsible for enhancing particle release in that only particles composed of Gag\* can be released efficiently. Alternatively, the modification of Gag may enhance virus release indirectly by rendering Gag\* unable to bind to Ubp thereby preventing the formation of 20 inhibitory Gag-Ubp complexes.

Recent experiments indicate that the matrix domain of HIV-1 Gag is required for Vpu-mediated enhancement of particle release (30). Based on these results, we speculate that UBP interacts with the matrix domain of 25 Gag. Preliminary experiments indicate that the interaction of Ubp with Gag may be mediated by the matrix/capsid junction and the p6 domain of Gag (data not shown). The fact that the matrix-capsid junction may be

involved in Gag-Ubp interaction raises the possibility that the negative effect of Ubp overexpression on particle release observed in our experiments was due to improper processing of Gag. However, this is unlikely  
5 because previous studies have shown that Gag processing does not affect virus release. Moreover, the effect of Vpu on virus particle release can occur in the absence of processing (21, 30). The roles of the matrix domain and Ubp/Gag interaction in Vpu-mediated enhancement of virus  
10 release are under investigation.

Ubp contains four tetratricopeptide repeat (TPR) motifs. TPRs are known to form a secondary structure that has been proposed to mediate interaction between two TPRs (26). Therefore, the TPR motifs of Ubp may mediate  
15 the interaction of Ubp with Vpu or Gag. Vpu and Gag do not contain TPR motifs. However, the TPRs of FKBP59 (34), Pxr1p (11), and a mouse homolog of PP5 (9) are directly involved in interactions with proteins that do not contain TPRs. Alternatively, the TPR motifs of Ubp  
20 could mediate interaction of Ubp with other cellular proteins involved in the enhancement of virus release. We are currently conducting experiments to identify the domains of Vpu, Gag, and Ubp that account for interactions between Ubp and Vpu or Gag.

25 The HIV-1 Gag protein interacts with cyclophilins A, B, and C, and cyclophilin A is incorporated into virus particles (15, 31, 46). Cyclophilins and FK506 binding proteins (FKBPs) comprise the immunophilin superfamily.

Immunophilins are involved in protein folding, and some act as intracellular receptors for the immunosuppressive drugs CsA and FK506. Ubp is probably not an immunophilin because it lacks an obvious binding site for CsA or

5 FK506. Moreover, Ubp is not detectably incorporated into virus particles (data not shown). However, one possible scenario for Ubp function is that Ubp "modifies" Gag by mediating the proper folding of the protein. The TPR proteins FKBP59 and CyP-40 are known to interact with  
10 heat shock protein 90 (hsp90), and these interactions are likely involved in the proper folding of steroid hormone receptors (34, 35).

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